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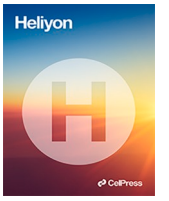

Research article

The Chinese herbal prescription JZ-1 promotes extracellular vesicle production and protects against herpes simplex virus type 2 infection *in vitro*

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ABSTRACT

Objective: Genital herpes, primarily caused by HSV-2 infection, remains a widespread sexually transmitted ailment. Extracellular vesicles play a pivotal role in host-virus confrontation. Recent research underscores the influence of Chinese herbal prescriptions on extracellular vesicle production and composition. This study aims to probe the impact of JieZe-1 (JZ-1) on extracellular vesicle components, elucidating its mechanisms against HSV-2 infection via extracellular vesicles.
Methods: The JZ-1's anti-HSV-2 effects were assessed using CCK-8 assay. Extracellular vesicles were precisely isolated utilizing ultracentrifugation and subsequently characterized through TEM, NTA, and Western Blot analyses. The anti-HSV-2 activity of extracellular vesicles was gauged using CCK-8, Western Blot, and immunofluorescence. Additionally, high-throughput sequencing was employed to detect miRNAs from extracellular vesicles, unraveling the potential antiviral mechanisms of JZ-1.
Results: Antiviral efficacy of JZ-1 was shown in VK2/E6E7, HeLa, and Vero cells. The samples extracted from cell supernatant by ultracentrifugation were identified as extracellular vesicles. In VK2/E6E7 cells, extracellular vesicles from JZ-1 group enhanced cell survival rates and diminished the expression of intracellular viral protein gD, contrasting with the inert effect of control group vesicles. Extracellular vesicles from JZ-1 treated Vero cells demonstrated a weaker yet discernible anti-HSV-2 effect. Conversely, extracellular vesicles of HeLa cells exhibited no anti-HSV-2 effect from either group. High-throughput sequencing of VK2/E6E7 cell extracellular vesicles unveiled significant upregulation of miRNA-101, miRNA-29a, miRNA-29b, miRNA-29c, and miRNA-637 in JZ-1 group vesicles. KEGG pathway analysis suggested that these miRNAs may inhibit PI3K/AKT/mTOR signaling pathway and induce autophagy of host cells to protect against HSV-2. Western blot confirmed the induction of autophagy and inhibition of AKT/mTOR in VK2/E6E7 cells with JZ-1 group extracellular vesicles treatment.
Conclusion: JZ-1 had an anti-HSV-2 efficacy. After JZ-1 stimulation, VK2/E6E7 cells secreted extracellular vesicles which protect host cells from HSV-2 infection. High-throughput sequencing

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showed that these extracellular vesicles contained a large number of miRNAs targeting PI3K/AKT/mTOR pathway. JZ-1 group extracellular vesicles could inhibit the activation of AKT/mTOR pathway and induce the host cells autophagy.

1. Introduction

Herpes simplex virus type 2 (HSV-2), also recognized as human herpesvirus type 2, is a double-stranded DNA virus. Genital herpes (GH) attributed to HSV-2 represents a highly prevalent sexually transmitted infection (STI), impacting over 16% of the population aged 15–49 years globally [1]. HSV-2 infection can result in genital herpes [2,3], perineal cluster herpes, causing symptoms such as burning pain, ulcers, and other lesions. Additionally, it has the potential to transmit vertically through the placenta during early pregnancy, leading to abortion or neonatal malformation [4]. Concurrently, HSV-2 infection serves as a significant co-factor in HIV infection, contributing to the transmission of HIV [5]. Given its substantial incidence and associated risks, the prevention and treatment of GH represent crucial global public health priorities. Presently, first-line drugs for genital herpes treatment primarily include nucleoside antiviral drugs such as famciclovir, valaciclovir, and acyclovir. While effective in inhibiting the gene synthesis of HSV-2, these drugs fall short in controlling disease recurrence, often causing adverse reactions in the digestive, nervous, and cutaneous systems. Additionally, drug resistance is escalating annually [6]. Consequently, there is a compelling need to explore more effective and safer anti-HSV-2 drugs.

Extracellular vesicles (EVs), characterized by a diameter ranging from 40 to 150 nm, are generated by various cell types and released into the extracellular space. They are ubiquitously present in bodily fluids like saliva and plasma, facilitating intercellular communication by acting as carriers for diverse proteins, mRNAs, and miRNAs to target cells [7]. Numerous studies have highlighted the crucial roles of extracellular vesicles in various domains, including tumor biology, infection, and immunity [8–10]. Notably, extracellular vesicles have demonstrated biological significance as intercellular transport vehicles during HSV-1 infection [11,12]. However, limited research has explored their function in HSV-2, which shares a genomic structure akin to HSV-1. Therefore, there is a justified need for further exploration into the role of EVs in HSV-2 infection.

MicroRNAs (miRNAs) are inherent non-coding RNAs with regulatory functions present in eukaryotes, typically measuring around 20 to 24 base pairs in length. MiRNAs usually regulate mRNA by recognizing and binding to it, degrading or repressing its translation [13]. Emerging evidence underscores the critical involvement of miRNAs in various viral infections, including HSV-2 [14], hepatitis B virus (HBV) [15], Epstein-Barr virus (EBV) [16], Zika virus [17], etc.

Genital herpes belongs to the category of “erosion of vulva” and “sore of vulvae” in traditional Chinese medicine (TCM). According to TCM theory, GH is due to the mixing of fire toxin of the liver meridian and damp heat, which converge on the genitals to form sores. The treatment based on TCM theory mainly focuses on clearing heat and toxins, and nourishing *Yin*. Patients with recurrent attacks should also strengthen their *vital qi*. The Chinese herbal compound JZ-1 is an external drug for the treatment of genital tract infectious diseases modified based on Yihuang decoction. JZ-1 is composed of ten Chinese herbs [18]. It has the efficacy of clearing heat and resolving dampness. In Tongji Hospital, JZ-1 has been used as an in-hospital preparation for the treatment of cervicitis, vaginitis, and condyloma acuminatum with remarkable clinical effects for many years. Previous experiments confirmed that JZ-1 effectively counters HSV-2 infection through various pathways. These pathways encompass the modulation of the Toll-like receptor signaling pathway, impeding the adhesion and penetration of HSV-2 into vaginal epithelial cells [19], and instigating autophagy in HSV-2 infected VK2/E6E7 cells while concurrently suppressing their pyroptosis [18,20]. Consequently, JZ-1 manifests a distinct anti-HSV-2 effect, indicating potential for wider clinical applications.

Motivated by the aforementioned considerations, this study focuses on extracellular vesicles as a potential mechanism for JZ-1 to facilitate the delivery of miRNA, thereby exerting an anti-HSV-2 efficacy. Our investigation employs VK2/E6E7 cells, the target cells for HSV-2 infection, Vero cells commonly used in virus research, and HeLa cells, a staple in extracellular vesicle research, to explore the impact of extracellular vesicles on HSV-2 infection.

2. Materials and methods

2.1. Cell culture

VK2/E6E7 cells were procured from the American Type Culture Collection (ATCC), Vero cells from the China Type Culture Collection (CTCC), and HeLa cells from the National Collection of Authenticated Cell Cultures. VK2/E6E7 cells were nurtured in Cn-TPR medium (CELLnTEC, Switzerland), while Vero and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cultures were maintained in a 5% CO2 atmosphere at 37 °C.

2.2. 3D-high performance liquid chromatography (3D-HPLC) of JZ-1

3D-high performance liquid chromatography was used to determine the primary components of JZ-1, as previously described [21].

2.3. Viral preparation

The HSV-2333 strain, sourced from Guangzhou Biotest Biotechnology Development Co., Ltd (Guangdong, China), underwent propagation utilizing Vero cells in high-glucose DMEM supplemented with 2% fetal bovine serum. The virus titers were assessed using the TCID50 method and adjusted to 1×10^7 TCID50/0.1 mL prior to utilization.

2.4. Cytopathic effect (CPE) inhibition assays

VK2/E6E7, Vero, and HeLa cells were uniformly seeded in 96-well plates and subjected to HSV-2, JZ-1, or extracellular vesicles. Assessment of cell viability was conducted using the Cell Counting Kit-8 (CCK-8, Abbkine Scientific Co., Ltd, China), and microscopic examination (Olympus, Japan) was performed after a 24-h incubation to observe any pathological changes.

2.5. Extracellular vesicle extraction

The cellular supernatant underwent sequential centrifugation steps at 300 g for 10 min and 2000 g for another 10 min. The resulting supernatant was further clarified through filtration with a 0.22 μm pore-sized filter, followed by a high-speed centrifugation at 120,000 g for 2 h. The resulting precipitate was collected, resuspended in PBS, and subjected to another centrifugation at 120,000 g for 2 h. After discarding the supernatant, the collected precipitate was resuspended in PBS to obtain extracellular vesicles. The extracted extracellular vesicles were then stored at −80 °C for future use.

2.6. Transmission electron microscopy (TEM)

Extracellular vesicles were immobilized with 2% phosphotungstic acid, applied onto 200-mesh copper carbon-supported grids, and subjected to observation through TEM (Hitachi, Japan; HT7800).

2.7. Nanoparticle tracking analysis (NTA)

Diluted extracellular vesicle samples were analyzed using the Zeta View particle tracking system (ZetaView, Particle Metrix, Germany, PMX110) for nanoparticle tracking analysis.

2.8. Western blot

Cellular and extracellular vesicle proteins were extracted using standard protocols. BCA protein assay kit (Beyotime, China) was employed to quantify protein concentrations. SDS-PAGE gel electrophoresis separated protein samples, subsequently transferred to NC membranes. The membranes were subjected to incubation with primary antibodies (TSG101, CD63, Alix, gD, GAPDH; Abcam, UK) (AKT, *p*-AKT; Proteintech, USA) (ATG5, mTOR, *p*-mTOR; CST, USA), followed by secondary antibodies. Scanning was performed using an Odyssey Infrared Laser Imaging system (LICOR Biosciences, USA). Antibody dilutions for TSG101, CD63, Alix, ATG5, *p*-AKT, AKT, *p*-mTOR, and mTOR were 1:1000, while gD and GAPDH antibodies were diluted at 1:10,000. Image J was utilized for result quantification.

2.9. Drug administration

The preparation process of JZ-1 was previously outlined [18]. Cells underwent a 24-h incubation with JZ-1, HSV-2, or extracellular vesicles.

2.10. Immunofluorescence

VK2/E6E7 cells underwent treatment, fixation, permeabilization, blocking, and subsequent incubation with a primary antibody (gD). Following this, cells were subjected to incubation with a secondary antibody and DAPI. The samples were observed using a fluorescence microscope (Olympus, Japan).

2.11. MiRNA sequencing

MiRNA library construction utilized The QIAseq miRNA Library Kit. Reads aligned to the miRBase database were quantified after quality assessment.

2.12. Statistical analysis

Statistical analysis for all data was performed using the *t*-test with GraphPad Prism 8. A significance threshold of $P < 0.05$ was applied to determine statistical significance.

3. Results

3.1. JZ-1's HPLC fingerprinting

The JZ-1's HPLC fingerprint, as previously presented in an earlier article [21], revealed the presence of nine primary chemical constituents: berberine, citric acid, caffeic acid, trigonelline, apocynin, luteolin, ferulic acid, taxifolin, and D-(−)-quinic acid.

3.2. JZ-1's cytotoxicity and anti-HSV-2 activity

Exploration of the JZ-1's non-cytotoxic concentration range in VK2/E6E7 cells, Vero cells, and HeLa cells was conducted, as depicted in Fig. 1A–C, E. The maximum non-cytotoxic concentrations were 12.5 mg/mL in VK2/E6E7 cells, 12.5 mg/mL in Vero cells, and 25 mg/mL in Hela cells. JZ-1 demonstrated antiviral effects in all three cell types, with effective concentration ranges of 1.5625–12.5 mg/mL, 3.125–12.5 mg/mL, and 12.5–25 mg/mL, respectively. The optimal antiviral concentration across all three cell types was determined as 12.5 mg/mL (Fig. 1B–D, F). Consequently, 12.5 mg/mL was selected as the JZ-1's concentration in this

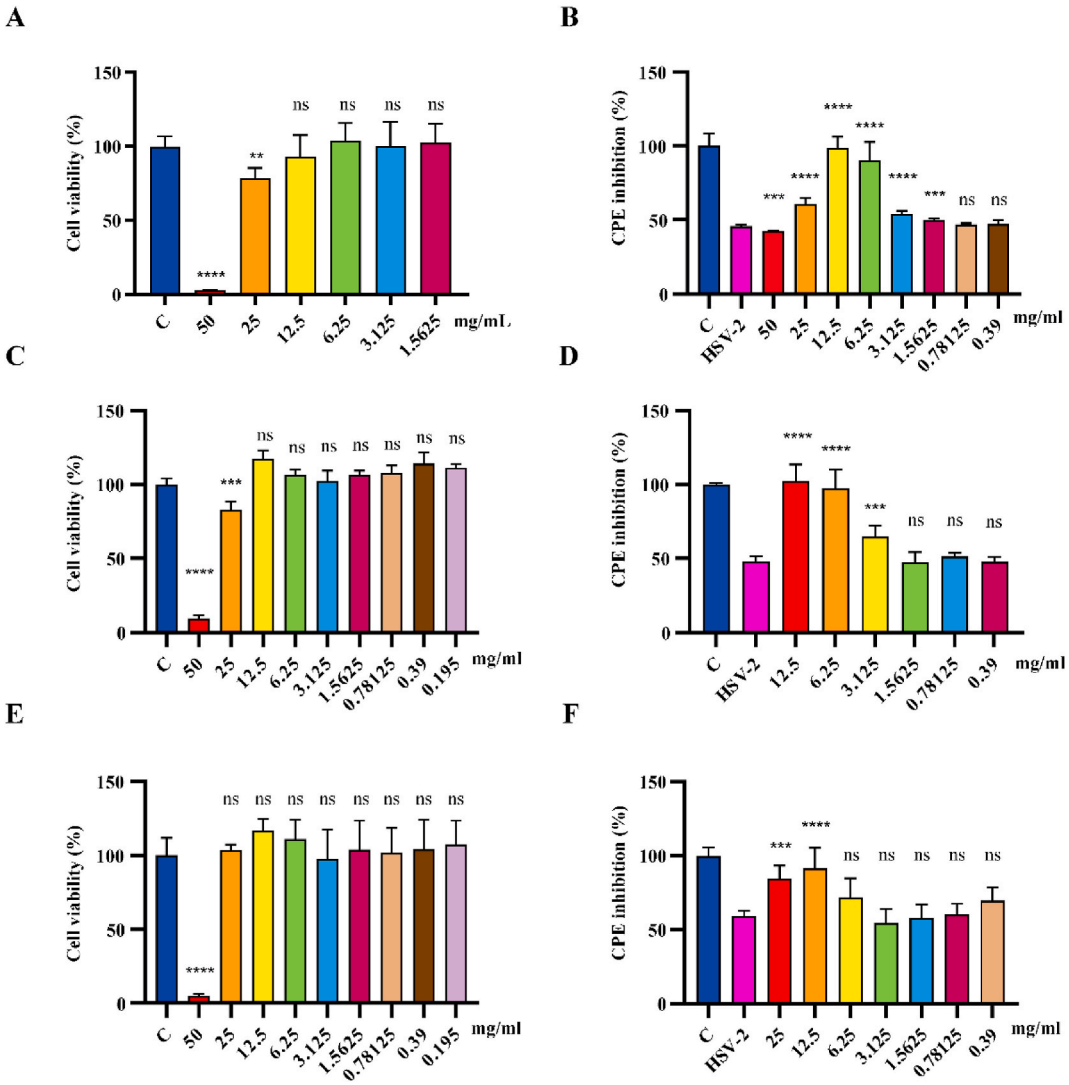

Fig. 1. JZ-1 has an anti-HSV-2 effect in vitro. A Cytotoxicity of JZ-1 in VK2/E6E7 cells was detected by CCK-8 method. B Assessment of the CPE inhibition levels for JZ-1 in VK2/E6E7 cells infected with HSV-2. C Cytotoxicity of JZ-1 in Vero cells was detected by CCK-8 method. D Assessment of the CPE inhibition levels for JZ-1 in Vero cells infected with HSV-2. E Cytotoxicity of JZ-1 in HeLa cells was detected by CCK-8 method. F Assessment of the CPE inhibition levels for JZ-1 in HeLa cells infected with HSV-2. For cell viability assessment, control cells without JZ-1 treatment were assigned values of 100% and the data were presented as mean ± S.D. For CPE inhibition assay, control cells without virus treatment were assigned values of 100% and the data were presented as mean ± S.D. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****); (n = 6).

project.

3.3. Collection and identification of extracellular vesicles

To explore the role of extracellular vesicles in JZ-1's antiviral effectiveness, we collected extracellular vesicles produced by VK2/E6E7, Vero cells, and HeLa cells, with or without JZ-1 stimulation, over a 24-h period. Extracellular vesicles obtained from every 100 mL of cell supernatant were resuspended in 100 μL PBS solution. Their characterization through transmission electron microscopy revealed vesicles with a dual membrane and diameters ranging from 40 to 150 nm (Fig. 2A). Nanoparticle tracking analysis demonstrated a size distribution between 40 and 150 nm, with the primary concentration around 100 nm (Fig. 2B). Western Blot analysis confirmed the presence of extracellular vesicle marker proteins, including CD63, Alix, and TSG101, in the collected samples (Fig. 2C), substantiating their classification as extracellular vesicles.

3.4. Anti-HSV-2 effect of extracellular vesicles

CCK-8 assay results indicated that extracellular vesicles from JZ-1-stimulated VK2/E6E7 cells, particularly at a high dose (8 μg/100 μL), effectively protected HSV-2-infected cells, while similar effects were less pronounced in Vero cells and absent in HeLa cells. However, the extracellular vesicles from control group showed no protective effect against HSV-2 in the three cells (Fig. 3A–C). Microscopic observations of cell morphology further supported these findings, demonstrating the restorative effects of JZ-1 group extracellular vesicles on infected VK2/E6E7 cells, while the effects were reversed by the extracellular vesicle internalization inhibitor Annexin V. The restorative effects of JZ-1 group extracellular vesicles were much weaker in Vero cells and absent in HeLa cells (Figs. S1A–C). Additionally, Western Blot analysis revealed a reduction in intracellular viral protein gD expression after treatment with JZ-1 group extracellular vesicles on infected VK2/E6E7 cells and Vero cells, but not HeLa cells (Fig. 3D–F). Further validation through immunofluorescence confirmed the substantial anti-HSV-2 effect of extracellular vesicles secreted by JZ-1-stimulated VK2/E6E7 cells (Fig. 3G).

3.5. High-throughput sequencing of miRNAs of extracellular vesicles in VK2/E6E7 cells

Given the antiviral impact of extracellular vesicles derived from the JZ-1 group of VK2/E6E7 cells as highlighted in the aforementioned investigation, we conducted high-throughput sequencing of miRNAs in EVs of VK2/E6E7 cells with and without JZ-1 stimulation, encompassing three replicates within each group. Subsequent statistical analysis was executed on miRNAs from the

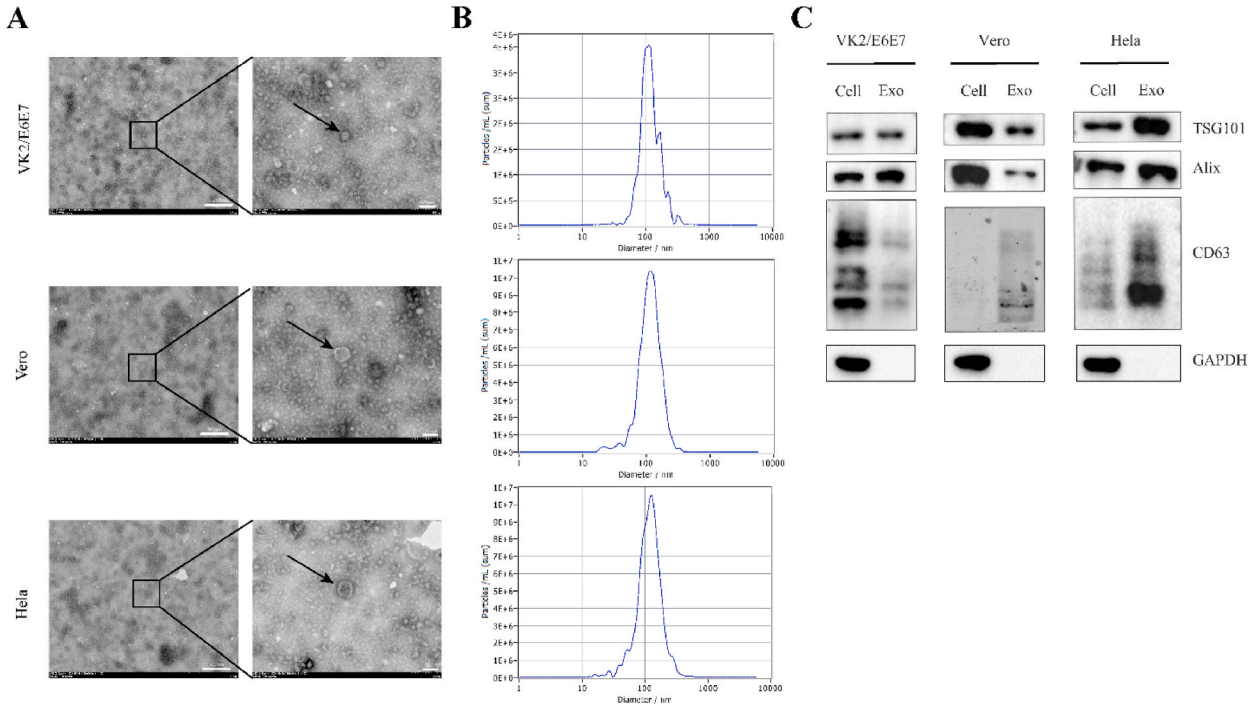

Fig. 2. Identifications of extracellular vesicles extracted from VK2/E6E7, Vero and HeLa cells in their native state. **A** Electron microscopy of extracellular vesicles extracted from VK2/E6E7, Vero and HeLa cells. **B** Nanoparticle tracking analysis of size distributions of extracellular vesicles extracted from VK2/E6E7, Vero and HeLa cells. **C** Western blotting analysis of exosomal or non-exosomal markers in the extracellular vesicles extracted from VK2/E6E7, Vero and HeLa cells. Complete, unaltered images are showcased in the Supplementary Materials.

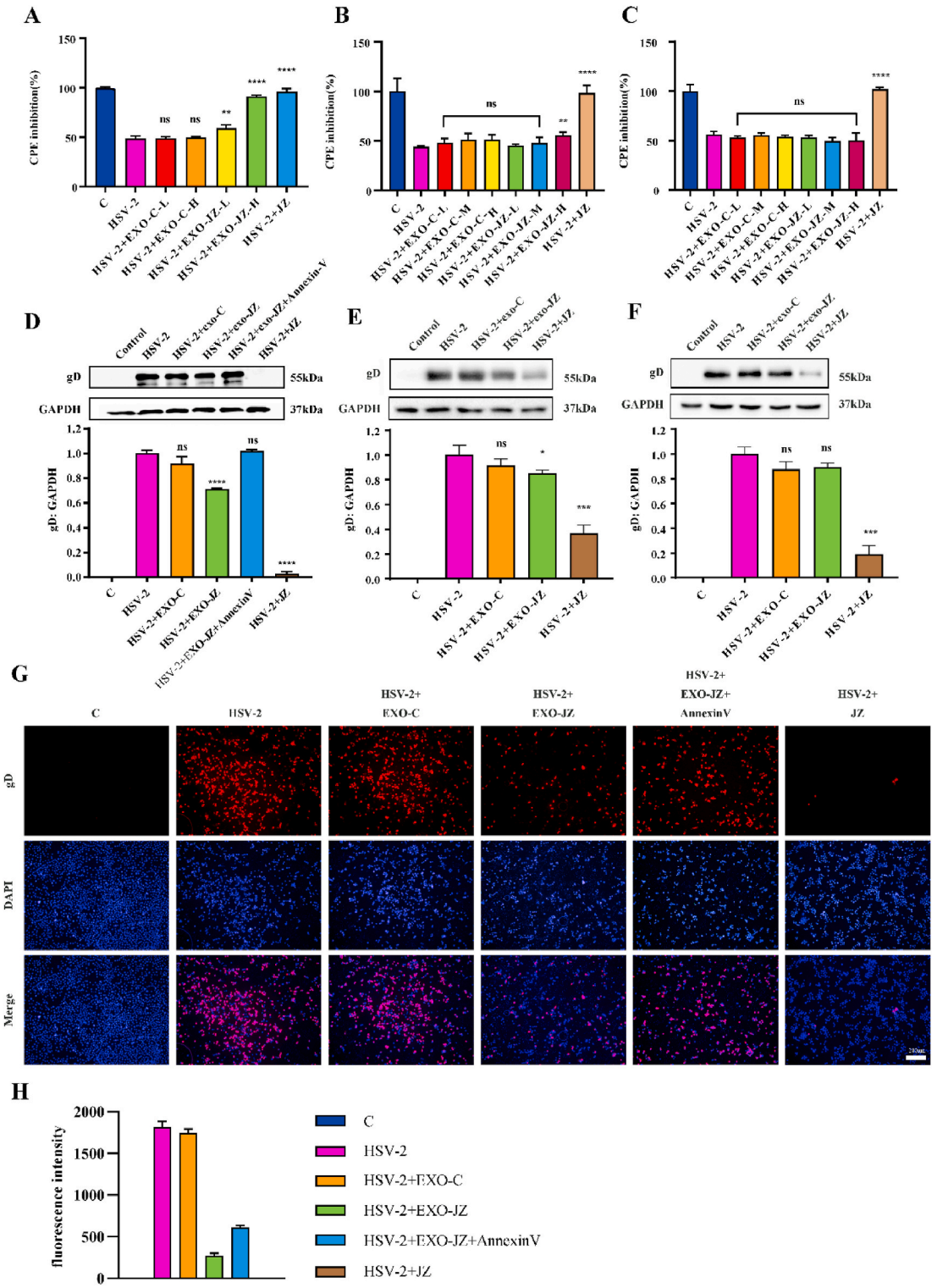

Fig. 3. The anti-HSV-2 effect of extracellular vesicles. **A** Anti-HSV-2 activity of extracellular vesicles (1 μg/100 μL, 8 μg/100 μL) of VK2/E6E7 cells was detected by CCK-8 method. **B** Anti-HSV-2 activity of extracellular vesicles (1 μg/100 μL, 5 μg/100 μL, 10 μg/100 μL) of Vero cells was detected by CCK-8 method. **C** Anti-HSV-2 activity of extracellular vesicles (1 μg/100 μL, 5 μg/100 μL, 10 μg/100 μL) of HeLa cells was detected by CCK-8 method. **D** gD level in VK2/E6E7 cells with extracellular vesicles (8 μg/100 μL) treatment was detected by western blotting. **E** gD level in Vero cells with extracellular vesicles (10 μg/100 μL) treatment was detected by western blotting. **F** gD level in HeLa cells with extracellular vesicles (10 μg/100 μL) treatment was detected by western blotting. Quantification of immunoblot for the ratio of gD to GAPDH was also shown. The ratios for non-

treated virus control cells (HSV-2) were assigned values of 1.0 and the data presented as mean ± S.D. (n = 3). Complete, unaltered images are showcased in the Supplementary Materials. **G** Immunoreactivity of gD was analyzed by immunofluorescence. The scale bar represents 200 μm. **H** Statistical analysis of the results presented in (G). $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****).

two groups. Calculating and visualizing the Pearson correlation coefficient for each sample demonstrated satisfactory repeatability between the groups (Fig. 4A). Next, we plotted the miRNAs whose expression differences between groups were consistent with $P < 0.05$ and $|\text{LogFC}| > 1$ into volcano plots and heat maps. In comparison to the control group's extracellular vesicles, 784 miRNAs were up-regulated, and 744 miRNAs were down-regulated in the extracellular vesicles of the JZ-1 group (Fig. 4B and C). Following this, KEGG enrichment analysis was conducted on the target genes of these miRNAs. It was observed that the target genes associated with the autophagy pathway exhibited significant enrichment (Fig. 4D). Previous research from our group indicated that JZ-1 induces autophagy in HSV-2 infected VK2/E6E7 cells by inhibiting the PI3K/AKT/mTOR signaling pathway, thereby restricting virus replication and infection expansion. Notably, miRNAs targeting the PI3K/AKT/mTOR signaling pathway, such as miR-101–3p, miR-29a-3p, miR-29b-3p, miR-29c-3p, and miR-637, were significantly upregulated in extracellular vesicles from the JZ-1 group compared to the control group (Table 1, Fig. 4E).

3.6. *Extracellular vesicles induce autophagy by inhibiting AKT/mTOR signaling pathway*

Based on the outcomes from high-throughput sequencing, our exploration into whether extracellular vesicles could regulate autophagy in VK2/E6E7 cells via the PI3K/AKT/mTOR signaling pathway involved assessing the autophagy marker protein ATG5 and the activation levels of AKT and mTOR in treated VK2/E6E7 cells. Illustrated in Fig. 5A and B, HSV-2 infection heightened the activation of the AKT/mTOR pathway while diminishing intracellular ATG5 content. In comparison to extracellular vesicles from the control group, the treatment with JZ-1 group extracellular vesicles impeded the activation of the AKT/mTOR pathway and reinstated the expression level of ATG5. Notably, this effect was reversed by the extracellular vesicle internalization inhibitor Annexin V. These findings further validate that extracellular vesicles from the JZ-1 group can inhibit the AKT/mTOR signaling pathway and induce cellular autophagy. This comprehensive investigation illuminates potential antiviral mechanisms associated with traditional Chinese medicine, specifically JZ-1, by modulating extracellular vesicles and miRNA-mediated pathways.

4. Discussion

Extracellular vesicles, initially identified by Johnstone in reticulocyte supernatant [22], arise from endosome membrane invagination, forming multivesicular bodies that merge with the cell membrane to release encapsulated vesicles known as EVs [23,24]. These vesicles harbor functional proteins, including ALG-2-interacting protein X (ALIX), tumor susceptibility gene 101 (TSG101), and the transmembrane protein superfamily (CD9, CD63, CD81, CD82, CD83), recognized as markers of extracellular vesicles. Moreover, extracellular vesicles contain various substances such as mRNAs, miRNAs, proteins, and lipids, capable of modulating diverse biological functions through endocytosis-mediated transfer to other cells [25].

Recent years have witnessed heightened interest in the intersection of extracellular vesicles and virus infection within virology research. Extracellular vesicles assume a dual role, influencing the virus-host conflict significantly. Viruses exploit extracellular vesicles to shield against immune attacks and enhance infection expansion [11,26]. For instance, during HSV-1 infection, extracellular vesicles released by infected cells impair the antiviral ability of uninfected bystander cells by conveying virions [11]. Notably, in recurrent herpes simplex keratitis (HSK), HSV-1 utilizes extracellular vesicles in tears as carriers for relapse and spread [26]. Conversely, extracellular vesicles serve as hosts' carriers, disseminating various cytokines, proteins, miRNAs, and substances to curtail viral infection and invasion [27,28]. Studies on HSV-1 reveal that host cell-secreted extracellular vesicles deliver miR-401, targeting and inactivating HSV-1 protein ICP4, effectively reducing the viral load in recipient cells [28]. Similarly, investigations into HBV demonstrate that serum extracellular vesicles, containing miR-574 from interferon-treated patients, exert an anti-HBV role by suppressing HBV replication and transcription in infected liver cells [27].

In our study, extracellular vesicles from JZ-1-stimulated VK2/E6E7 cells exhibited anti-HSV-2 effects. Drawing from existing research, we postulate that miRNAs transported within extracellular vesicles play a pivotal role. High-throughput sequencing revealed significant changes in the expression of miRNAs targeting autophagy. While Vero cells' extracellular vesicles demonstrated similar effects, they were comparatively weaker than those from VK2/E6E7 cells, and HeLa cells' extracellular vesicles showed no anti-HSV-2 effect. Due to challenges in obtaining, separating, and concentrating extracellular vesicles, the exploration of antiviral effects at higher concentrations remains an avenue for future investigation.

High-throughput sequencing miRNAs in extracellular vesicles released by VK2/E6E7 cells, with or without JZ-1 treatment, revealed noteworthy differential expression of miRNAs targeting autophagy. Intriguingly, our previous research indicated that JZ-1 induces autophagy in HSV-2-infected VK2/E6E7 cells by suppressing the PI3K/AKT/mTOR signaling pathway, thereby hindering the expansion of virus infection [18,29]. Autophagy, a conserved intracellular process, involves encapsulating damaged organelles or misfolded proteins in a double-membrane structure that fuses with lysosomes to form autolysosomes. Autophagy plays a crucial role in maintaining cellular homeostasis, including protecting cells from nutrient deprivation and microbial infections. ATG5, a pivotal molecule in autophagosome formation, reflects the extent of autophagy [30]. mTOR, a key autophagy regulator, acts as a checkpoint in its negative regulation. The activation of the PI3K/AKT/mTOR pathway suppresses autophagy [31]. Notably, miR-101–3p, miR-29a-3p, miR-29b-3p, miR-29c-3p, and miR-637 were significantly upregulated in extracellular vesicles from the JZ-1 group

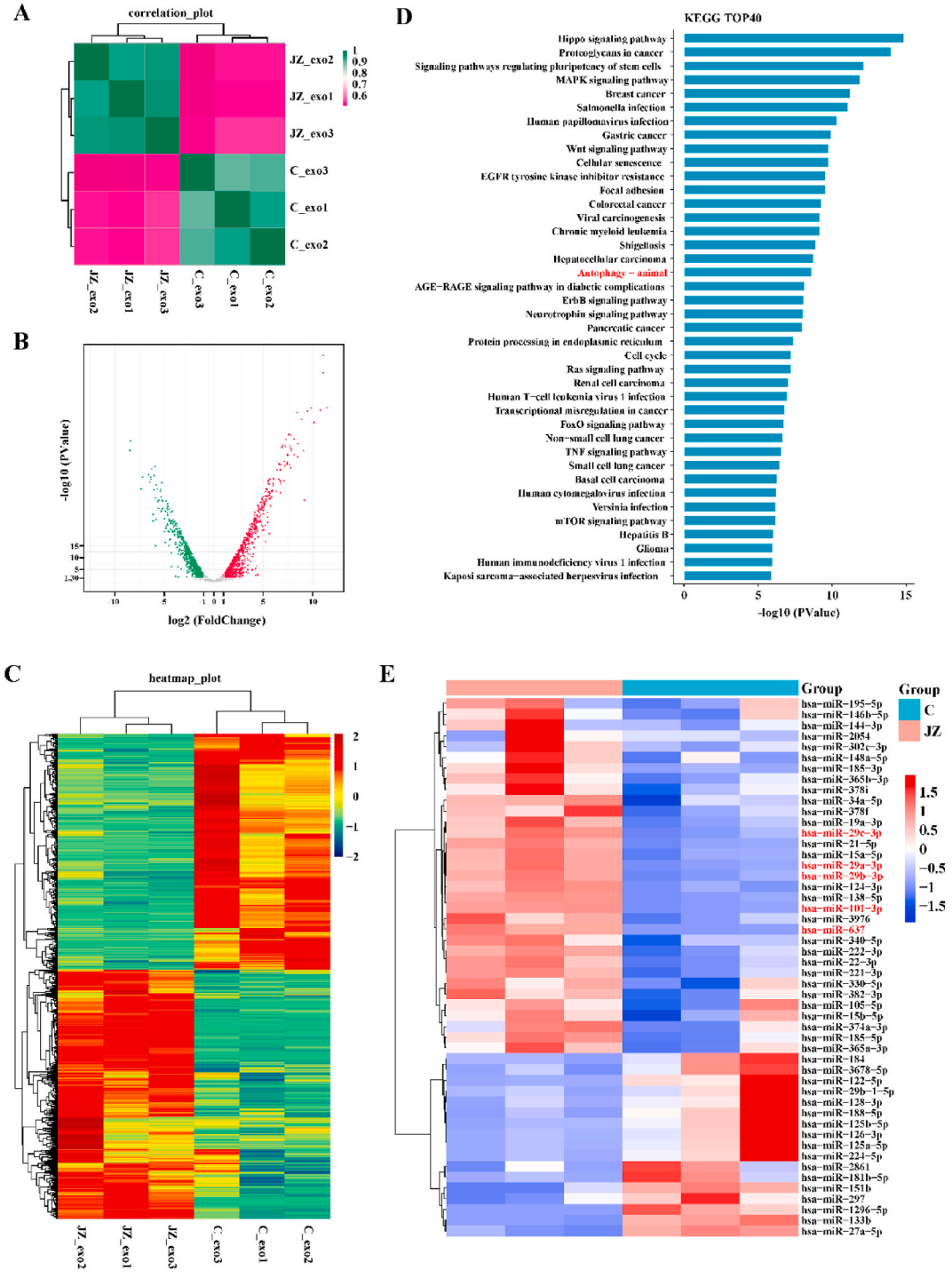

Fig. 4. High-throughput sequencing of extracellular vesicles of VK2/E6E7 cells. **A** Heat map shows the hierarchically clustered Pearson's correlation matrix resulting from comparing the miRNAs expression values for extracellular vesicle samples of control and JZ-1 groups. **B** Volcano plot shows upregulated and downregulated extracellular vesicle miRNAs derived from VK2/E6E7 cells with or without JZ-1 treatment. Red dots indicate upregulated (LogFC >1, $P < 0.05$) while green dots indicate downregulated (LogFC < −1, $P < 0.05$). **C** Hierarchical clustering of differentially expressed miRNAs in extracellular vesicles of JZ-1 and control group. FPKM values are log2-transformed and then median-centered by each gene. **D** KEGG functional pathways of genes targeted by differentially expressed miRNAs in extracellular vesicles of JZ-1 and control group. **E** Heat map shows the differentially expressed miRNAs targeting PI3K/AKT/mTOR pathway in extracellular vesicles of JZ-1 and control group.

Table 1
The information of miRNAs targeting PI3K/AKT/mTOR pathway in extracellular vesicles secreted from VK2/E6E7 cells with or without JZ-1 treatment.

miRNA	P value	FDR	LOG2FC	mRNA Target
hsa-miR-637	2.29E-31	4.61E-30	4.572592	AKT
hsa-miR-101–3p	4.42E-24	5.54E-23	4.301703	mTOR
hsa-miR-29a-3p	7.92E-21	7.68E-20	3.553933	AKT
hsa-miR-29b-3p	4.35E-18	3.51E-17	3.115682	AKT
hsa-miR-29c-3p	2.55E-12	1.34E-11	2.250693	AKT
hsa-miR-19a-3p	1.18E-10	5.47E-10	2.014135	AKT
hsa-miR-3976	1.59E-07	5.58E-07	1.778592	AKT
hsa-miR-144–3p	0.013645	0.024471	1.720559	AKT
hsa-miR-34a-5p	3.27E-05	8.72E-05	1.57517	AKT
hsa-miR-185–5p	8.03E-06	2.33E-05	1.377604	AKT
hsa-miR-15b-5p	0.001011	0.002226	1.083068	AKT

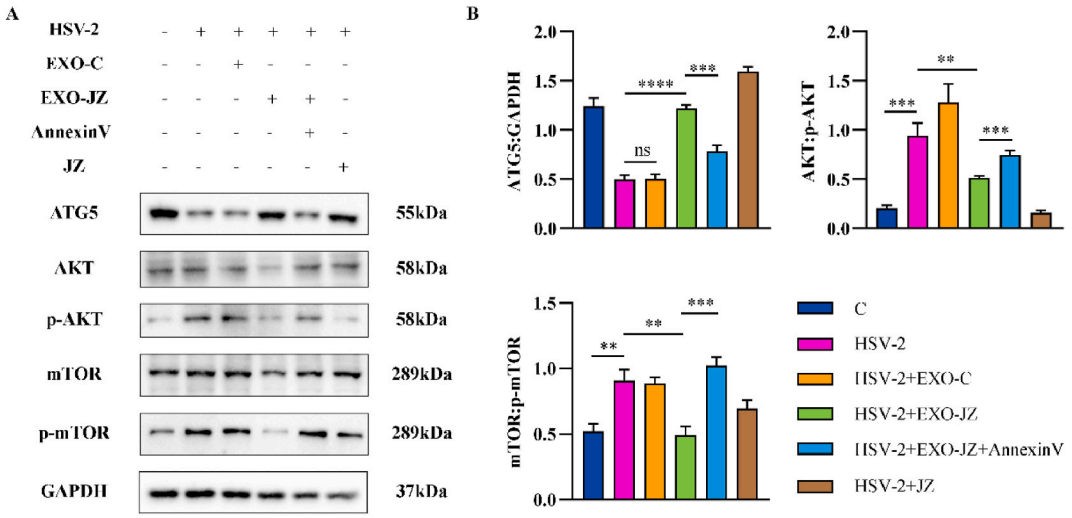

Fig. 5. JZ-1-stimulated extracellular vesicles induced autophagy via the PI3K/AKT/mTOR pathway. **A** VK2/E6E7 cells were treated with HSV-2 in the presence of extracellular vesicles (8 μg/100 μL) or JZ-1 for 24 h, and the ATG5, Akt, *p*-Akt, mTOR, and *p*-mTOR levels were determined by western blotting. Complete, unaltered images are showcased in the Supplementary Materials. **B** Results from the quantification of the Western blot shown in (A). $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****).

compared to the control group. These miRNAs have been confirmed to target components of the PI3K/AKT/mTOR pathway. Research has shown that mTOR is targeted by miR-101–3p, and its transfection in endothelial cells significantly reduces mTOR levels, promoting endothelial cell proliferation [32]. In gastric cancer cells, exogenous expression of miR-101–3p significantly reduces mTOR expression, exerting potential tumor inhibition effects [33]. Numerous studies have confirmed that miR-29 targets AKT and reduces its expression. In various cancer types, miR-29 has demonstrated anti-proliferative and anti-metastatic effects by targeting AKT [[34], [[35–38]. Additionally, miR-637 has been reported to target and reduce AKT expression in different tissues, exerting anti-tumor effects [39–41]. In our experiments, extracellular vesicles from the JZ-1 group reduced AKT and mTOR activation and promoted autophagy in HSV-2 infected VK2/E6E7 cells. We hypothesize that JZ-1 stimulates VK2/E6E7 cells to secrete extracellular vesicles containing these miRNAs, which are delivered to HSV-2-infected cells. This induction of autophagy occurs through the inhibition of the PI3K/AKT/mTOR pathway, ultimately restricting HSV-2 infection. This study provides a novel perspective on JZ-1's efficacy against HSV-2 infection and unveils the impact of traditional Chinese medicine on extracellular vesicles, enriching our scientific understanding of the antiviral properties of traditional Chinese medicine.

5. Conclusion

This study unequivocally establishes JZ-1 as a potent agent against viral infections, showcasing its dual capability in directly combating viruses and eliciting the secretion of extracellular vesicles endowed with anti-HSV-2 properties. Through comprehensive high-throughput sequencing analysis and validation, we discerned that the antiviral effect of JZ-1 might be closely related to the delivery of multiple miRNAs encapsulated in extracellular vesicles. The extracellular vesicles released by VK2/E6E7 cells with JZ-1 treatment hindered the PI3K/AKT/mTOR signaling pathway, prompting autophagy in host cells, and subsequently manifested anti-HSV-2 effects. This novel revelation not only expands our understanding of JZ-1's antiviral mechanisms but also sheds light on the

broader application of TCM in antivirus research. The intricate interplay between JZ-1, extracellular vesicles, and miRNAs unveils promising avenues for future investigations and underscores the rich potential of traditional Chinese medicine in advancing antiviral therapeutic strategies.

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CRediT authorship contribution statement

Wenjia Wang: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ximing Jin:** Validation, Project administration, Investigation. **Qingqing Shao:** Validation, Project administration. **Tong Liu:** Project administration. **Tianli Liu:** Investigation. **Xinwei Zhao:** Project administration. **Lijun Xu:** Resources. **Wen Gao:** Writing – review & editing. **Liu Hu:** Funding acquisition. **Zhuo Chen:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27019.

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